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MODIFICATION OF THE NUTRITIONAL COMPOSITION OF ARTEMIA BY INCORPORATION OF POLYUNSATURATED FATTY ACIDS USING MICRO-ENCAPSULATED DIETS

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ABSTRACT

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The development of a partially defined artificial encapsulated diet for *Artemia* nauplii is described and feeding experiments are used to demonstrate how this diet may be modified to determine the nutritional requirements of *Artemia*. Analysis shows that fatty acids such as 20:5 ω 3 and 22:6 ω 3 supplied in the encapsulated diet appear in the tissues of *Artemia* nauplii fed on these diets, thus improving the nutritional value of *Artemia* as a live food for marine fish and invertebrate larvae, which have a dietary requirement for long chain polyunsaturated fatty acids.

INTRODUCTION

Previous work has shown that the technique of micro-encapsulation may be used to introduce semi-defined artificial diets as particulates to *Artemia* which successfully promote growth and survival of the nauplii (Jones et al., 1974; Jones and Gabbott, 1976). The present work describes the development of a further refined artificial microencapsulated diet for *Artemia* and demonstrates how this may be used to examine dietary requirements of *Artemia* for sterols, nucleic acids, and certain amino acids. In addition the dietary requirements of *Artemia* nauplii for certain fatty acids are examined and a capacity to alter the level of polyunsaturated fatty acids (PUFA) in the nauplius by feeding artificial diets is also demonstrated.

This capacity is considered important since recent work (Howell, 1979; Scott and Middleton, 1979) has questioned the adequacy of *Artemia* nauplii and rotifers as live foods to promote high growth and survival levels in marine larvae. Moreover, Watanabe et al. (1978, 1980) have clearly demonstrated that *Artemia* from different sources vary considerably in their nutritional

value as food to marine fish larvae. They have been able to show that it is the level of certain long chain PUFA of the $\omega 3$ series, notably 20:5 $\omega 3$, in newly hatched *Artemia* nauplii, that governs their food value to marine fish. A dietary requirement for long chain PUFA has been found in red sea bream, *Chrysophrys major*, rockfish *Sebastiscus marmoratus*, globefish *Fugu rubripes rubripes* (see Kanazawa et al., 1979), turbot (Owen et al., 1975; Cowey et al., 1976), plaice (Owen et al., 1972), prawns *Penaeus japonicus* (Jones et al., 1979a), *Penaeus setiferus*, *P. aztecus*, and *P. duorarum* (Bottino et al., 1980) and in the oysters *Crassostrea virginica* (Trider and Castell, 1980) and *C. gigas* (Langdon and Waldock, 1981).

MATERIALS AND METHODS

The microcapsules used in experiments were of the nylon cross-linked protein-wall type described by Chang et al. (1966), although their preparation was modified to reduce toxicity (Jones et al., 1976). They were prepared using a Silverson Homogeniser to reduce the capsule diameter to between 5 and 10 μm , and fed at a concentration of 1.0×10^8 capsules/l of seawater in all experiments.

The diets supplied in the microcapsules were either homogenised fresh whole chicken egg or modifications of the partially defined artificial diet given in Table I, which is based on the diet B developed by Kanazawa et al. (1977). To this diet were added the nucleic acids RNA and DNA and putrescine as these have previously been identified as growth promoting agents for *Artemia* (Provasoli, 1975). Details of the vitamin and mineral mixes are given by Kanazawa et al. (1977). The *Tapes* oil used was extracted from *Tapes philippinarum*, the short-neck clam, and cod liver oil was supplied by Alembic Products Ltd. Manchester, Great Britain. Control groups of animals were either starved or fed on live food, *Chaetoceros curvisetus*, at a density of 2.0×10^8 cells/l seawater.

All the *Artemia* used in the experiments were from the same batch of San Francisco strain and were hatched under strong light in seawater at 25–27°C. Feeding trials were carried out with 375 nauplii/l using UV-irradiated seawater filtered through a 0.2 μm membrane filter. Experiments were carried out at 21–22°C for 18 days in 1 l beakers supplied with aeration and a paddle stirring system (Jones et al., 1979a) to keep the microcapsules in suspension. Each experiment was replicated and growth and survival of *Artemia* was monitored every other day. Results are expressed using mean total length and the growth index devised by Provasoli and D'Agostino (1979).

The total lipid fraction of the diets and whole bodies of *Artemia* was extracted by the method of Folch et al. (1957) using 2:1 v/v chloroform : methanol containing 0.01% w/v of the anti-oxidant 2,6-di-*tert* butyl-*p*-cresol. Total lipids were separated into neutral and polar lipid fractions by silicic acid column chromatography (Christie, 1973), and fatty acid methyl esters

TABLE I

Composition of partially defined artificial diets for encapsulation used in feeding experiments with *Artemia*. The mineral and vitamin mixtures are as detailed in Kanazawa et al. (1977). Ten grammes of diet was added to 50 ml water and pH adjusted to 7. Other diets used were prepared from the above diet as follows: cholesterol 'free', cholesterol omitted; nucleic acid 'free', RNA and DNA omitted; amino acid 'free', methionine and tryptophan omitted; lipid free, cod liver oil omitted; *Tapes* oil, 10 g *Tapes* oil substituted for 10 g cod liver oil; soybean oil, 10 g soybean oil substituted for cod liver oil.

Ingredients	g/100 g dry diet
Glucose	5.5
Sucrose	10.0
Starch	4.0
Casein (lipid and vitamin free)	50.0
Glucosamine	0.8
Na-citrate	3.0
Na-succinate	3.0
Cholesterol	0.5
Cod liver oil	10.0
Mineral mixture	8.5
Vitamin mixture	5.5
Putrescine	0.02
L-methionine	1.0
L-tryptophan	0.5
Alk. hydr. RNA	0.6
Alk. hydr. DNA	2.4

prepared using boron trifluoride in methanol (Morrison and Smith, 1964). Fatty acid methyl esters were separated by gas liquid chromatography on a Hewlett-Packard 5720A equipped with a glass column (3 m × 2 mm i.d.) packed with 10% SP 1000 on Chromosorb W. HP, 80-100 mesh (Phase Sep. Deeside, Great Britain). Fatty acids were identified using commercially available standards (C.S. Chromatography Services, Merseyside, Great Britain) and graphical techniques (Ackman, 1972).

RESULTS

Table II compares the survival and growth of *Artemia* fed on an encapsulated partially defined artificial diet (Table I) and whole egg, which has usually been included as a diet in previous experiments (Jones et al., 1979b), with control groups fed on live algae and starved. Although *Chaetoceros* sustained the highest survival rate, the highest growth rate was achieved on the artificial diet which produced adults in 18 days and which produced better survival and growth than encapsulated egg.

TABLE II

Comparison of survival and growth of *Artemia* over a period of 18 days fed on *Chaetoceros curvisetus*, artificial encapsulated diets, and starved controls

Diet	Survival (%)	Mean body length (mm)	Growth index ^a (\pm SE)
Partially defined	34.9	5.65	13.0 \pm 0
Whole egg	27.2	3.44	11.0 \pm 0
<i>Chaetoceros curvisetus</i>	48.0	4.17	11.7 \pm 0.3
Starved control	0 ^b	1.08	3.0 \pm 0

^a Growth index developed by Provasoli and D'Agostino (1969).

^b Result after 6 days.

TABLE III

Comparison of survival and growth of *Artemia* over a period of 18 days fed on artificial encapsulated diet, and the same diet minus nucleic acids, methionine and tryptophan, cholesterol. Growth index as in Table I.

Diet	Survival (%)	Mean body length (mm)	Growth index (\pm SE)
Partially defined	34.9	5.65	13.0 \pm 0
Cholesterol free	5.1	3.20	11.0 \pm 0
Nucleic acid free	20.0	4.19	11.8 \pm 0.73
DL-methionine and L-tryptophan free	8.0	3.41	11.0 \pm 2.6

In a second series of experiments various components of the partially defined diet (Table I) were omitted in turn, whilst other dietary constituents remained constant, to test the effect of their absence on the growth and survival of *Artemia*. Table III shows that the omission of cholesterol produced the most serious effect, reducing survival to only 5.1% after 18 days, although survivors reached growth index 11. The removal of the additional amounts of the amino acids methionine and tryptophan resulted in a similar trend (Fig. 2), but the omission of the nucleic acids RNA and DNA appears to have less effect on growth and survival, although final survival levels are well below those achieved on the complete artificial diet.

In a third series of experiments (Table IV) the effect of different dietary lipids upon growth and survival of *Artemia* was tested. In addition the fatty acid composition of the dietary lipid was determined (Table V). Survival rates appear best on the original partially defined diet which contained cod liver oil, although when this is replaced by *Tapes* oil there is little change (Table IV). Both cod liver oil and *Tapes* oil contained the PUFA, 20:5 and 22:6. Similarly growth rates on both these lipid sources are high, with *Artemia* reaching the adult stage on both diets. In contrast survival on a

partially defined diet containing soybean oil, which is lacking in PUFA, was low, only marginally better than survival on a lipid free diet. Surprisingly the survivors of the groups fed on lipid free and soybean lipid diets achieved a growth index of over 11 (Table IV), although both groups failed to achieve adulthood.

The effect of the different fatty acid composition of the diet (Table V) on the proportions of fatty acids in the polar and neutral lipid fractions of *Artemia* nauplii is shown in Table VI. Certain striking changes take place in the fatty acid composition of *Artemia*. It is apparent that if the diet contains a large amount of a particular fatty acid this will be reflected in the fatty acid composition of *Artemia* nauplii fed on that diet. Soybean oil contains a large proportion of 18:2 ω 6 (47.4% of the total fatty acids determined) (Table V), and results in a marked increase in the level of this fatty acid in both the polar and neutral lipid fraction of *Artemia* fed on this diet, when compared to *Artemia* fed on diets which are low in 18:2 ω 6 (Table VI).

TABLE IV

Comparison of survival and growth of *Artemia* over a period of 18 days on artificial encapsulated diets containing different lipids. Growth index as Table I

Diet	Survival (%)	Mean body length (mm)	Growth index (\pm SE)
Cod liver oil	34.9	5.65	13.0 \pm 0
Tapes oil	28.5	6.92	14.4 \pm 0.93
Soybean oil	12.0	4.14	11.6 \pm 0.71
Lipid free	4.3	3.48	11.2 \pm 1.06

TABLE V

The fatty acid composition of the dietary lipid used in feeding experiments

Fatty acid	Cod liver oil total lipid	Tapes total lipid	Soybean total lipid	Chaetoceros curvisetus	
				neutral lipid	polar lipid
14:0	8.1	3.4	0.1	9.0	23.7
16:0	15.3	25.9	13.3	5.0	6.8
16:1 ω 7	14.3	7.6	0.1	14.5	13.5
18:0	2.6	5.9	4.4	—	—
18:1 ω 9 + ω 7	21.0	6.8	20.2	—	—
18:2 ω 6	1.8	0.5	47.4	9.2	4.3
18:3 ω 3	0.2	0.9	8.0	—	—
20:4 ω 6	—	3.3	—	—	7.6
20:5 ω 3	10.9	13.6	—	45.9	—
22:6 ω 3	2.2	13.2	—	—	—

Data expressed as percentage of total fatty acids present

TABLE VI

The fatty acid composition of *Artemia* nauplii after 18 day feeding experiments

Fatty acid	Newly hatched <i>Artemia</i> nauplii	<i>Artemia</i> nauplii starved for 4 days	Diet				Soybean oil
			<i>Chaetoceros curvisetus</i>	Lipid free	Cod liver oil	<i>Tapes</i> oil	
<i>Polar lipid</i>							
12:0	0.4	11.1	tr	tr	tr	0.6	0.6
14:0	0.7	tr	tr	2.5	tr	1.8	2.0
16:0	12.6	8.3	15.5	13.6	9.4	9.4	10.6
16:1 ω 7	3.6	1.6	19.4	6.8	7.2	5.6	5.6
18:0	7.1	8.6	11.0	10.6	9.1	10.3	10.3
18:1 ω 9 +							
ω 7	33.6	36.5	30.6	43.2	43.7	40.1	35.5
18:2 ω 6	6.9	5.5	2.8	8.2	7.8	5.5	5.5
18:3 ω 3	20.0	15.6	3.9	7.0	6.9	6.3	6.3
20:4 ω 6	tr	4.5	tr	1.0	3.3	4.1	4.1
20:5 ω 3	1.0	1.4	12.7	1.6	9.2	8.0	8.0
22:6 ω 3	tr	tr	tr	tr	0.1	0.7	0.7
<i>Neutral lipid</i>							
12:0	tr	4.6	tr	6.1	tr	4.5	4.5
14:0	1.3	9.2	8.6	9.2	4.1	8.6	8.6
16:0	15.8	21.1	19.3	18.7	23.0	19.8	19.8
16:1 ω 7	5.3	5.6	27.4	7.5	5.5	7.5	7.5
18:0	3.2	6.1	2.6	8.2	14.3	8.1	8.1
18:1 ω 9 +							
ω 7	26.5	23.4	19.8	30.0	46.1	37.2	31.5
18:2 ω 6	7.1	3.9	2.3	5.8	3.7	4.0	15.3
18:3 ω 3	27.2	11.3	tr	1.8	1.1	1.6	1.6
20:4 ω 6	3.4	tr	tr	tr	tr	tr	tr
20:5 ω 3	tr	tr	4.5	tr	tr	tr	tr
22:6 ω 3	tr	tr	tr	tr	tr	tr	tr

Data expressed as percentage of total fatty acids present.

tr = trace.

Likewise *Chaetoceros curvisetus* contains the highest proportion of 20:5 ω 3 (approximately 46% of the total fatty acids present when compared to the other diets) (Table V), and when fed results in a significant increase in the levels of 20:5 ω 3 in *Artemia* polar and neutral lipid fractions. However, although 20:4 ω 6 was present in the algae in significant amounts there was no marked effect in the trace levels of 20:4 ω 6 in the nauplii. The pertinent effect of the defined diet was seen in *Artemia* fed cod liver oil and *Tapes* oil, both of which contain 20:5 ω 3 and 22:6 ω 3. This resulted in a measurable increase in the 20:5 ω 3 and 22:6 ω 3 levels in the polar lipids of nauplii fed on these oils when compared to nauplii fed on a diet of soybean oil.

which contains fatty acids no longer or more unsaturated than 18:3 ω 3, or on a lipid free diet.

DISCUSSION

Provasoli and D'Agostino (1969), using an artificial medium in axenic growth studies on *Artemia*, found that cholesterol, nucleic acids and certain vitamins were essential ingredients for normal development. However, as their defined diets relied upon many of the ingredients being supplied as solubles in the culture medium itself, it was impossible to determine essential amino acids and energy sources such as soluble polysaccharides as these would have had to be added at toxic concentrations (Provasoli, 1975). Previous work (Jones et al., 1974; Jones and Gabbott, 1976) has shown that semi-defined diets may be supplied to *Artemia* in the form of microcapsules, and present work shows that further refined diets may also be fed successfully in this way. Although the present experiments were not conducted axenically, it has been shown that aseptic techniques may be applied to nutritional studies using microencapsulated diets (Jones et al., 1976).

The role of vitamins in animal nutrition is now considered classic knowledge, but the specific dietary requirements for certain other biochemical constituents in marine animals remains intriguing. The sterol requirement of crustaceans has been recognised for some time, since marine crustaceans are not capable of synthesising cholesterol (Goad, 1976) but can convert ingested sterols into cholesterol (Kanazawa et al., 1976). Teshima and Kanazawa (1971) have shown that *Artemia* can convert dietary ergosterol into cholesterol. Yet a further confirmation of a requirement for dietary sterols in Crustacea is seen in the poor survival and growth of *Artemia* fed on a cholesterol-free diet.

Similarly the poor survival of *Artemia* nauplii on the nucleic acid free diet is not unexpected, since the nucleic acid requirement of *Artemia* for optimum growth is now clear from both biochemical and nutritional experiments (Provasoli, 1975). *Artemia* is incapable of synthesising the purine ring *de novo* (Clegg et al., 1967) and so has an absolute need for a purine source, such as adenylic, inosinic or guanylic acid, in the diet. On the other hand *Artemia* can synthesise the pyrimidine nucleotides, albeit at a limiting rate, since the growth rate and survival of *Artemia* nauplii is increased by the addition of pyrimidine sources such as cytosine monophosphate or uridine monophosphate in the diet (Hernandorena, 1979). This situation is similar to the effect of certain long chain PUFA on marine animals to be discussed later. It would seem that *Artemia* nauplii are best reared on a diet that contains both purine and pyrimidine nucleotides in small amounts.

The low survival and growth rates observed on the diet in which methionine and tryptophan were omitted (Table III) are perhaps an indication of the levels at which these amino acids should be supplied as they are of course present in small amounts in casein, the major protein source in the defined diet.

Watanabe et al. (1978, 1980) have shown that the long chain PUFA composition of newly hatched *Artemia* varies considerably depending on the place of origin and even from time to time at the same location. These authors have also demonstrated that *Artemia* lacking in long chain PUFA form a poor nutritional source for certain marine fish larvae. Kanazawa et al. (1979) have shown that 18:3 ω 3 can be converted rapidly into 20:5 ω 3 and 22:6 ω 3 in the trout, but that this ability apparently decreases in the order trout, ayu, eel, prawn, red sea bream, rockfish and globefish. Marine flatfish such as plaice (Owen et al., 1972), turbot (Owen et al., 1975) must also be low down on this list. It now seems quite clear both from experiments using radioactive precursors and nutritional studies that numerous marine fish, notably all top carnivores, cannot convert C₁₈ into C₂₀ and C₂₂ PUFA at a significant rate (Owen et al., 1975; Fujii and Yone, 1976; Kanazawa et al., 1979), and this also seems to hold true for other marine species such as prawns (Jones et al., 1979a; Bottino et al., 1980) and oysters (Trider and Castell, 1980). The present results indicate that *Artemia* cannot synthesise them at a reduced rate, since the nauplii grow better on diets containing the PUFA 20:5 ω 3 and 22:6 ω 3 (Table IV). Presumably 20:5 ω 3 is of most importance since this acid is present in all strains of newly hatched *Artemia* whereas 22:6 ω 3 is absent (Table V; Enzler et al., 1974; Gallagher and Brown, 1975; Watanabe et al., 1978).

It is interesting that in *Artemia* fed on diets containing cod liver oil and *Tapes* oil, both of which contain 22:6 ω 3, the levels of 22:6 ω 3 in the polar lipid fraction increased somewhat from just trace levels in newly hatched nauplii, but remained in trace quantities only in nauplii raised on soybean oil which lacks 22:6 ω 3. Similar results were obtained by Watanabe et al. (1978, 1980) when they fed *Artemia* on yeast rich in 22:6 ω 3.

When *Artemia* nauplii are fed algae such as *Chaetoceros curvisetus* (present work) and *Chlorella* (Watanabe et al., 1980), or artificial diets containing cod liver oil or *Tapes* oil which are rich in 20:5 ω 3 (Table II), there is also a significant increase in the level of this fatty acid in the polar lipid fraction (Table III and Watanabe et al., 1980). Finally it can be seen from Tables V and VI that, as in prawns (Colvin, 1976; Guary et al., 1976), when *Artemia* are fed seed oils such as soybean oil rich in 18:2 ω 6 then this fatty acid accumulates in the tissue lipid.

It is now well established that the fatty acid composition of lipids from the tissues of higher animals reflects the fatty acid composition of dietary lipids and this is now generally assumed to be also true for lower species including invertebrates (Holland, 1978). Watanabe et al. (1978, 1980) have recently shown how this knowledge may be successfully applied to fish larval culture by feeding live foods containing high levels of long chain PUFA to strains of *Artemia* deficient in these fatty acids, and then using the enriched *Artemia* as food to promote the growth of marine fish larvae which have a dietary requirement for long chain PUFA. In the present work we have been able to demonstrate that there is now a choice in the first stage

of larval culture. Cultures of algae or yeast cells rich in long chain PUFA may be fed to *Artemia*, or the *Artemia* may be fed artificial encapsulated diets of chosen nutritional composition with regard to the level of vitamins, cofactors etc., and the particular long chain PUFA required by the marine larvae under culture may be incorporated. Further, by adjusting the composition of the artificial encapsulated diet fed to *Artemia* nauplii, it should now be possible to compensate for variations in the nutritional value of *Artemia* from different stocks, thus ensuring that all nauplii whatever their origins present a nutritionally adequate diet for marine fish and invertebrate larvae.

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